GDC-0853 inhibits both wild type and C481S BTK variants while preserving NK cell mediated ADCC

Sean D. Reiff, Daphne Guinn, Bonnie Harrington, Carolynn Cheney, Amy Lehman, Rose Mantle, Lisa Smith, Amy J. Johnson, John C. Byrd, Jennifer A. Woyach

Abstract

Bruton's tyrosine kinase (BTK) has recently emerged as an attractive therapeutic target in hematologic malignancies. Ibrutinib, the first in class BTK inhibitor, improves survival and is well tolerated in patients. Here, we investigate a novel BTK inhibitor, GDC-0853, to evaluate its efficacy in chronic lymphocytic leukemia (CLL). GDC-0853 is unique among BTK inhibitors in that it utilizes a novel allosteric binding site, different from the C481 kinase domain to which ibrutinib irreversibly binds. Like ibrutinib, GDC-0853 blocks B cell receptor signaling, modestly reduces viability, prevents stromal and cytokine induced survival, and limits activation in CLL lymphocytes through the inhibition of BTK. We found that due to its novel site of action, GDC-0853 also inhibits C481S mutated BTK in an in vitro system. GDC-0853 is more selective for BTK than is ibrutinib which also irreversibly inhibits interleukin-2 inducible kinase (ITK), a Tec kinase responsible for T and NK cell activation. The selectivity of GDC-0853 preserves ITK function in T and NK cells. Unlike ibrutinib, upper-physiologic concentrations of GDC-0853 had no effect on T cell activation or NK cell antibody dependent cell mediated cytotoxicity (ADCC). Our results indicate that the GDC-0853 may be an effective therapy for use in CLL as monotherapy or in combination with anti-CD20 antibodies, especially among the emerging population of patients who are resistant to first generation BTK inhibitors.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia, with an annual incidence of over 16,000 individuals in the United States. Bruton's tyrosine kinase (BTK) has recently emerged as an attractive therapeutic target in hematologic malignancies. BTK serves to potentiate signaling from proximal receptors. Although its function is identical between healthy and malignant B cells, BTK is over-expressed at the transcript level and constitutively active in CLL (add references). BTK dependent pathways are initiated at the B cell receptor (BCR), toll-like receptors (TLRs), chemokine receptors and a variety of cytokine receptors which contribute to canonical and non-canonical NF-kB signaling. Mouse models which overexpress BTK through a B cell specific promotor are associated with increased mortality due to a CLL-like disease. B lymphocytes from these mice are hyperresponsive to BCR stimulation and demonstrate resistance to Fas-mediated apoptosis. Taken together, these findings implicate BTK as a driver of CLL pathology.

Ibrutinib, the first in class BTK inhibitor, is approved for the treatment of CLL, mantle cell lymphoma, and waldenstrom macroglobulinemia. Ibrutinib is well tolerated in patients and has shown outstanding clinical activity in both relapsed and treatment-naïve CLL, even in those with high risk cytogenetic abnormalities. The clinical success of ibrutinib validates BTK inhibition as a powerful therapeutic model in hematologic malignancies. Ibrutinib irreversibly binds to and inhibits BTK at its C481 active site, a domain conserved among TEC family kinases. Despite the widespread success of ibrutinib, current BTK inhibitor therapy is imperfect and resistance has been reported. Acquired resistance to ibrutinib is most commonly mediated by mutation of the C481 amino acid of BTK to which ibrutinib irreversibly binds. These mutations prevent ibrutinib's irreversible inhibition of BTK and greatly diminish its potency.

Additionally, nonspecific recognition of the TEC family active site by ibrutinib leads to the inhibition of alternative targets including ITK, a tyrosine kinase responsible for T and NK cell activation. Inhibition of ITK by ibrutinib has been shown to inhibit specific T helper cell populations and limit the efficacy of antibody dependent cell mediated cytotoxicity (ADCC) by NK cells. Ibrutinib's inhibition of ITK prevents this BTK inhibitor from being effectively combined with clinically established antibody therapies which rely upon NK cell mediated ADCC.

Here we characterize a novel, reversible BTK inhibitor, GDC-0853, with a unique mode of inhibition. Rather than inhibiting BTK through its C481 active site like ibrutinib, GDC-0853 utilizes an allosteric binding pocket to exert its inhibitory effects. Because GDC-0853 does not rely upon C481, we hypothesized that this compound would maintain efficacy in ibrutinib resistance mediated by C481S mutations. This will establish for the first time in CLL, the potential of circumventing ibrutinib resistance through the use of an alternative BTK inhibitor. As well, we hypothesized that because it does not bind ITK through the conserved C481 site, that GDC-0853 would not limit T cell activation or NK cell mediated ADCC.

Methods

Subject Population and Lymphocyte Isolation

Unless otherwise noted all studies used cells isolated from the whole blood of CLL patients at our institution who consented to an IRB-approved tissue procurement protocol or who were included on IRB approved clinical trials of ibrutinib in CLL. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood through ficoll density gradient centrifugation.

Specific leukocyte populations were negatively selected using Rosette-sep isolation from whole blood or Easy-sep negative selection from PBMCs.

Cell Culture and Drugging

To specifically inhibit the irreversible targets of ibrutinib, ibrutinib treatment occurred for 1 hour followed by washout in which the cells were pelleted and resuspended in fresh 10% FBA RPMI-1640 media. Cells treated with vehicle or GDC-0853 were similarly pelleted and resuspended in 10% FBS RPMI-1640 media containing vehicle or GDC-0853. Experiments which occurred over several days were treated with daily drugging and media replacement.

BCR Signaling Immunoblot

CLL cells treated with BTK inhibitor were stimulated by spinning onto a six-well plate coated with anti-IgM antibody at 10ug/mL. Following fifteen minutes of stimulation, cells were lysed using a standard protein lysis buffer containing phosphatase and protease inhibitors. Immunoblot experiments were conducted by SDS-PAGE. Blots were probed with appropriate primary antibodies and HRP-conjugated secondary antibodies and visualized with chemiluminescent substrate on X-ray film. Densitometry analysis was performed using AlphaView software.

Viability Analysis

Primary CLL cells were assessed for survival 48hrs following treatment with BTK inhibitor. Viability was defined as the percentage of PBMCs staining negative for 7AAD. For co-culture experiments, stromal cells were stably transfected with green fluorescent protein (GFP) allowing us to selectively gate on our GFP-negative CLL population in addition to gates based on cell size. Samples with viabilities below 40% at 48hrs were omitted from analysis due to their rapid

rate of ex-vivo apoptosis and unpredictable behavior in vitro. All viability measurements were acquired with a Beckman Coulter FC3000 flow cytometer.

CpG induced activation

Following BTK inhibitor treatment CLL cells were stimulated with 3.2ug CpG/mL. Subsequent expression of CD86 was measured by determining Mean Fluorescence Intensity (MFI) after 48 hrs. Staining intensity of CD86-PE was measured using a Beckman Coulter FC3000 flow cytometer.

T cell immunoblot

Healthy human T cells were stimulated for 45 minutes with a combination of 2ug/mL plate bound anti-CD3 and 10ug/mL soluble anti-CD28. Nuclear and cytoplasmic lysates were collected using a ThermoScientific NE-PER kit and analyzed by SDS-PAGE as previously described.

NK cell mediated ADCC

Effector NK cells were isolated from Leukopaks obtained through the American Red Cross and incubated with target CLL cells loaded with radioactive Cr51 at an effector to target ratio of 25:1. Following treatment of purified NK cells with vehicle, GDC-0853, or ibrutinib for 1 hour, CLL cells were incubated with antibody at a concentration of 10ug/mL and co-cultured with NK cells to allow for lysis. After 4 hours of co-culture, supernatant was collected and measured for radiation using a PerkinElmer Wizard2 gamma counter. These radiation measurements were scaled according to a no NK cell co-culture group with baseline CLL lysis and a detergent treated CLL group with complete lysis.

Statistical Analysis

A mixed effect model provided by A.L. was used for statistical analysis.

Results

GDC-0853 effectively inhibits BTK

To investigate the effect of GDC-0853 on BCR signaling, purified PBMCs from CLL patients were treated with drug for 1 hour and then stimulated with anti-IgM antibody for 15 minutes. Treatment with GDC-0853 inhibited BTK phosphorylation and downstream activation of PLCg2, AKT and ERK similar to the inhibition seen with ibrutinib (Figure 1). Inhibition was dose-dependent but was present even at low concentrations of GDC-0853.

BTK inhibitors have been shown to possess modest levels of direct cytotoxicity against malignant CLL B cells. To characterize the cytotoxic potential of GDC-0853, primary CLL cells were treated with BTK inhibitor and assessed for changes in viability. After 48 hours, the viability of primary CLL cells treated with GDC-0853 was 61.8%, a statistically significant reduction compared to the vehicle treated viability of 68.4% (Figure 2A). To determine if GDC-0853 maintained efficacy in a model of the bone marrow microenvironment, primary CLL cells were co-cultured with the human HS5 stromal cell line. GDC-0853 remained cytotoxic in the presence of HS5 stromal cells (Figure 2B). Furthermore, absolute viability following treatment with GDC-0853 did not differ between the media and co-culture conditions ($p=0.386$), suggesting that GDC-0853 may abrogate the survival benefit of the bone marrow microenvironment in addition to its direct cytotoxicity. To test this we sought to determine if GDC-0853 negatively affected survival in the presence of various stimulatory cytokines. Anti-IgM, CD40L, and BAFF are potent activators of both the canonical and non-canonical NFkB

pathways. As expected from our stromal co-culture data, we found that GDC-0853 mitigated survival in response to these stimulatory factors (Figure 2C). GDC-0853's ability to maintain cytotoxicity in the presence of these activators demonstrates that it exerts broad efficacy across many stimulatory receptors.

TLR9 recognizes unmethylated CpG antigens commonly found in bacterial DNA. Stimulation by this nonspecific molecular pattern initiates a BTK dependent signaling cascade which results in B cell activation. Surface expression of CD86 is increased following ligation of TLR9 with CpG and is a common means of measuring B cell activation. We found that increases in CD86 expression induced by CpG are decreased 40% following treatment with GDC-0853 (Figure 3). This effect was similar to ibrutinib and demonstrates that GDC-0853 limits the activation of malignant B cells in CLL.

GDC-0853 inhibits C481S BTK mutants

Mutation of its C481 BTK binding site is the most common acquired form of resistance to ibrutinib. Plasmids containing wild type or C481S BTK were stably transfected into HEK 293T cell lines. As expected, ibrutinib was only able to inhibit wild-type BTK and was ineffective against the C481S mutated BTK variant (Figure 4). However, GDC-0853 was found to inhibit both wild-type and C481S mutated BTK to a similar degree.

GDC-0853 lacks ITK inhibition and preserves NK cell mediated ADCC

In much the same way that B lymphocytes rely upon BTK to promote survival and activation, T and NK cells rely upon ITK and other Tec family kinases to elicit their cytotoxic effects. Stimulation through the T cell receptor (TCR) constituent CD3 and the co-stimulatory molecule CD28 provides a potent activating signal in T cells. IKBa is phosphorylated following TCR

stimulation resulting in nuclear translocation of NFAT and subsequent changes in gene expression. Because of its unique BTK binding site we hypothesized that, in contrast to ibrutinib, GDC-0853 would lack recognition of the conserved Tec family kinase domain which ibrutinib nonspecifically targets. This would be expected to spare the function of T and NK cells. In healthy donor T cells, ibrutinib inhibits the phosphorylation of IKBa and NFAT nuclear localization. Treatment with GDC-0853, however, preserves T cell activation following TCR stimulation (Figure 5A).

Antibody therapies have been used to prolong patient survival in CLL for decades. However, combinations of ibrutinib with anti-CD20 antibodies like rituximab have not been shown to be more effective than ibrutinib alone. This is presumably due to ibrutinib rendering antibody therapy ineffective through its inhibition of ITK. Knowing that GDC-0853 preserves ITK activity in T cells, we tested whether GDC-0853 would be effective in combination with antibody therapies. We found significant differences in antibody mediated NK cell ADCC between GDC-0853 and ibrutinib (Figure 5B). Combinations of GDC-0853 with alemtuzumab, rituximab, ofatumumab, and obinutuzumab possessed 24.8, 27.8, 28.3, and 26.1 percent greater absolute cytotoxicity compared to ibrutinib, respectively.

Discussion

BTK inhibition has been shown to be a successful therapeutic strategy in CLL as well as other B cell malignancies. As the clinical usage of BTK inhibitors continues to increase, so does the incidence of resistance and need for alternative therapies. Therefore, developing BTK inhibitors like GDC-0853 with unique modes of action is of high importance. Herein, we have described a

novel BTK inhibitor with a unique BTK binding site that has the potential to circumvent resistance to ibrutinib and combine successfully with antibodies.

Comparable to the established BTK inhibitor ibrutinib, GDC-0853 inhibits BTK mediated functions contributing to CLL pathology. GDC-0853 effectively blocks BCR mediated survival signaling via its direct repression of BTK and indirect inhibition of PLCg2, AKT, and ERK. By targeting these anti-apoptotic targets, GDC-0853 is expected to reduce survival in primary CLL cells. Indeed, we were able to show that GDC-0853 is directly cytotoxic in vitro to primary CLL B cells. Furthermore, we have shown that this compound maintains cytotoxicity even in models of the protective bone marrow microenvironment. Cytotoxicity in HS5 co-culture experiments indicates that our investigational compound may directly abrogate important cytokine induced survival. In addition to inhibition of the BCR pathway and direct cytotoxicity, BTK inhibitors also reduce the activation of CLL B cells via inhibition of TLR signaling. GDC-0853 was able to inhibit CLL activation at a level comparable to ibrutinib, indicating that this compound may be an effective clinical BTK inhibitor.

Resistance to ibrutinib is recurrently mediated by mutation of its C481 BTK binding site. Most commonly, this takes the form of a C481S missense mutation which converts ibrutinib from a potent irreversible inhibitor to a low potency reversible inhibitor. Due to its distinct site of action, GDC-0853 was hypothesized to be effective in C481S mutated BTK variants, and we show this to be the case in vitro. Critically, there is data to suggest that even in the presence of the C481S mutation, the BCR pathway remains functional, therefore there is rationale to target the mutation with an alternative BTK inhibitor such as GDC-0853. Given that patients who develop resistance to ibrutinib experience rapid disease progression the need for effective

therapies in these populations is great. Our data provide evidence that BTK inhibition can still be achieved even in the context of ibrutinib resistance in patients.

CD20 monoclonal antibody therapies are known to increase survival in CLL when combined with active agents, so the combination of a BTK inhibitor with a CD20 monoclonal antibody is appealing. Ibrutinib in combination with clinically relevant antibodies used in CLL has been effective, but it is unclear whether the combination is superior to single agent therapy, and in vitro data suggests that it should not be. By simultaneously blocking BTK mediated survival signaling and actively promoting CLL clearance through immune effector cells, combinations of GDC-0853 with antibody therapy may be more synergistic than combinations with ibrutinib. While it has been proposed that ITK inhibition by ibrutinib may be beneficial due to the suppression of immunosuppressive Th2 responses, proof that ibrutinib potentiates beneficial Th1 responses is lacking. The consequences of BTK specific and ITK specific inhibition on CLL biology warrant further investigation.

GDC-0853 represents an exciting development in BTK directed therapy. This compound performs comparably to ibrutinib in terms of its ability to mitigate BCR signaling, CLL survival, and B cell activation. Additionally, we have validated the potential of circumventing ibrutinib resistance by inhibiting an allosteric BTK binding site. Due to its novel site of action, GDC-0853 possesses fewer alternative targets thereby preserving T cell activation and NK cell mediated ADCC. These data show for the first time the potential of reversible BTK inhibitor therapy and justify further investigation into GDC-0853 and related agents.

Acknowledgements

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Author Contributions

S.D.R. designed and conducted experiments, analyzed data, generated figures, interpreted results, and wrote the manuscript. D.G. provided necessary reagents. C.C., R.M., L.S, B.H. and generated data. A.L. performed the statistical analysis. A.J.J supervised research, JCB and JAW planned the project, supervised the research, analyzed data, and obtained funding for the research work. All authors approved reviewed and modified drafts of the manuscript and approved the final version. **Disclosure of Conflicts of Interest**

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Figure Legends

Figure 1. BCR signaling is inhibited by GDC-0853 Lysate from 1E7/mL primary CLL cells were treated with BTK inhibitor or DMSO for 1 hour. After stimulation, total lysates were subjected to SDS-PAGE to investigate BCR signaling. (A) The representative sample demonstrates nanomolar inhibition of BTK and PLCg2 and dose dependent decreases in the activation of AKT and ERK. (B-E) Densitometry data from five CLL patients were combined to compose average patterns of activation by anti-IgM and repression by BTK inhibitor. Ibrutinib was used as a positive control to model BTK inhibition.

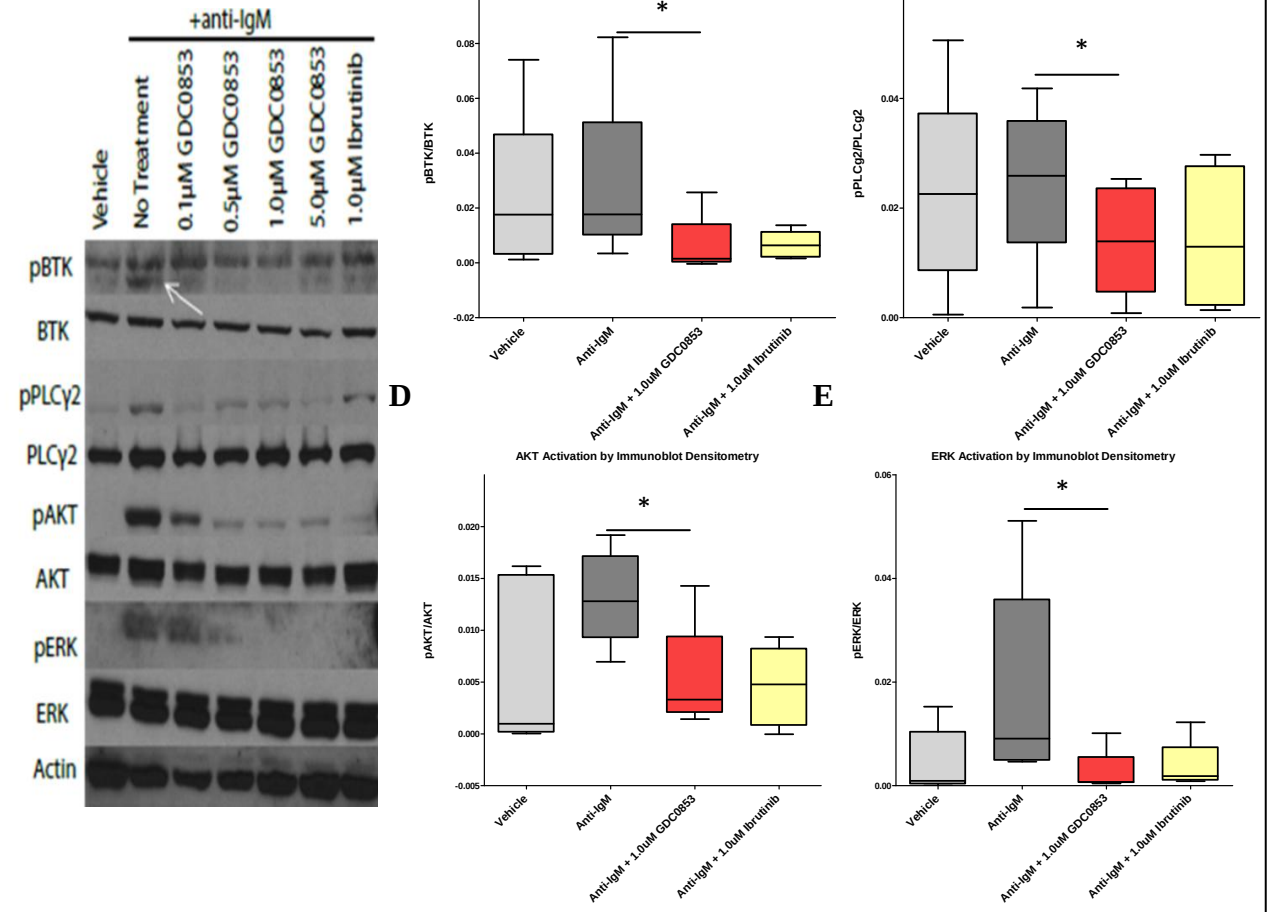
Figure 2. GDC-0853 is directly cytotoxic to CLL B cells and limits stromal induced survival Cultures of 1E7/mL primary CLL cells treated with 1um BTK inhibitor or DMSO were maintained in 10% complete RPMI media for 48hrs and assessed for viability. (A) The raw CLL viability was determined by 7AAD staining and read by flow cytometry (N=16). (B) GFP-HS5 stromal cells were plated and permitted to reach 50-75% confluence before being co-cultured with 1E7/mL primary CLL. Both populations were then drugged and viability was measured at 48hrs (N=9). (C) BTK inhibitor treated CLL cells at 1E7/mL were stimulated by 10ug/mL soluble anti-IgM, 1ug/mL CD40L, or 50ng/mL BAFF for 48hrs (N=8). Viability was normalized to an unstimulated vehicle condition.

Figure 3. TLR mediated activation is reduced by GDC-0853 1E7/mL primary CLL cells were treated with BTK inhibitor and stimulated with 3.2ug/mL of CpG-ODN (N=15). After 48hrs cells were stained with CD86-PE antibody and measured for fluorescence by flow cytometry. MFI results were normalized to untreated and unstimulated paired samples.

Figure 4. GDC-0853 effectively inhibits both wild type and C481S mutated BTK variants The HEK 293T BTK null cell line was stably transfected with either wild type or C481S mutated

BTK. Following BTK inhibitor treatment these cell lines were lysed and subjected to SDS-PAGE analysis.

Figure 5. ITK dependent activation of T and NK cells is preserved by GDC-0853 (A) Following 1 hour treatment with BTK inhibitor or vehicle, 1E6/mL healthy human T cells were activated by antiCD3/antiCD28 stimulation. Nuclear and cytosolic lysates were collected and analyzed by SDS-PAGE. (B) Cr51 labeled CLL B cells treated with antibody therapies were co-cultured with BTK inhibitor treated NK cells at a target to effector ratio of 1:25. Following four hours of lysis supernatant from these co-cultures was collected and analyzed for levels of radioactive Cr51. 2 separate CLL patients were each co-cultured with 2 allogenic NK cell donors for a total of 4 replicates.



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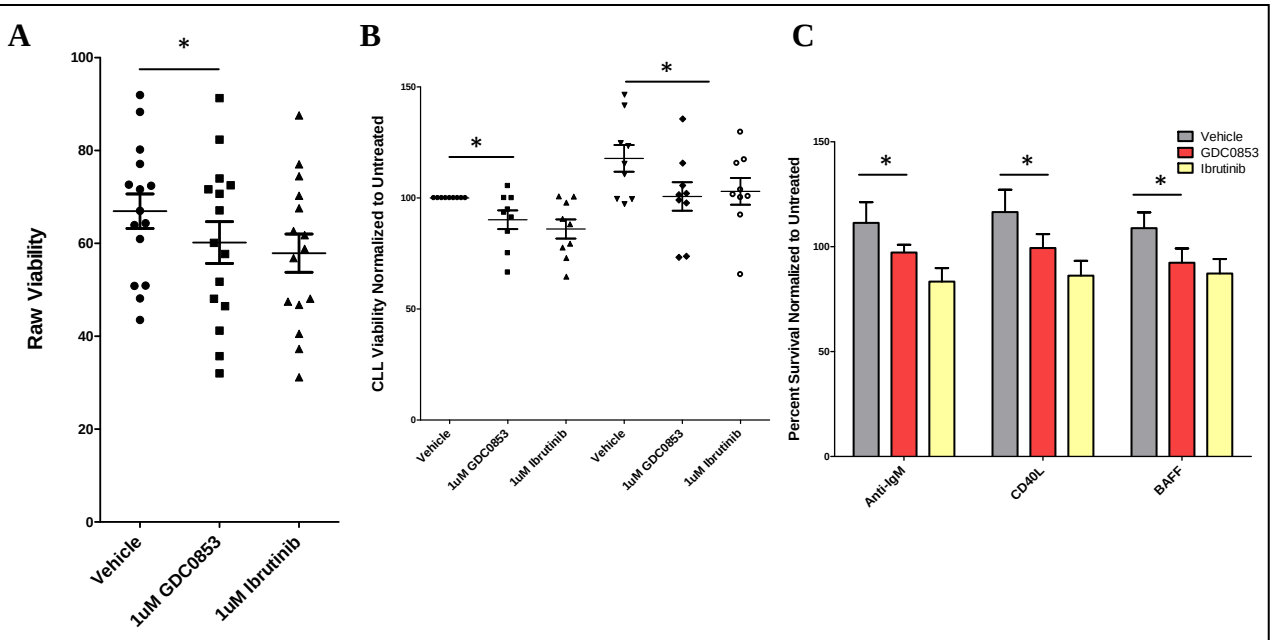


Figure 2. GDC-0853 is directly cytotoxic to CLL B cells and limits stromal induced survival

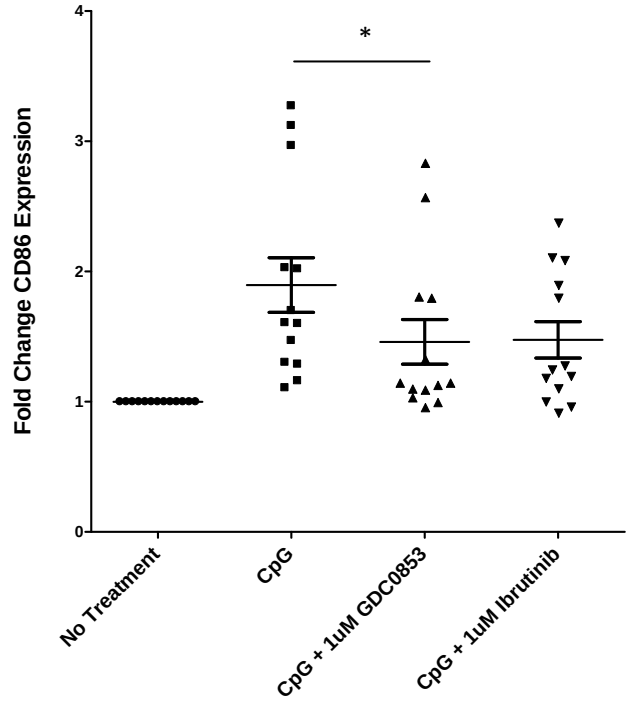
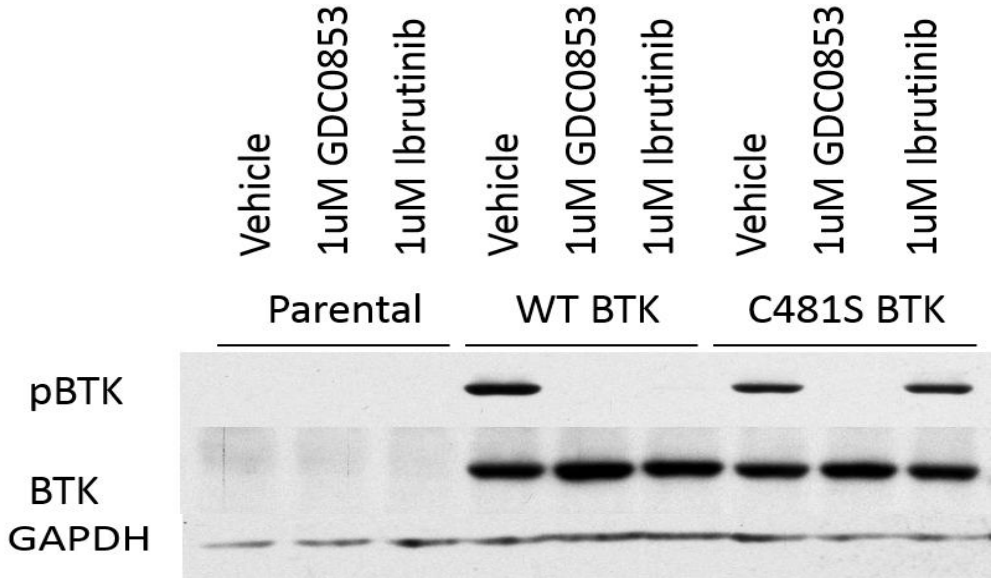


Figure 3. TLR mediated activation is reduced by GDC-0853



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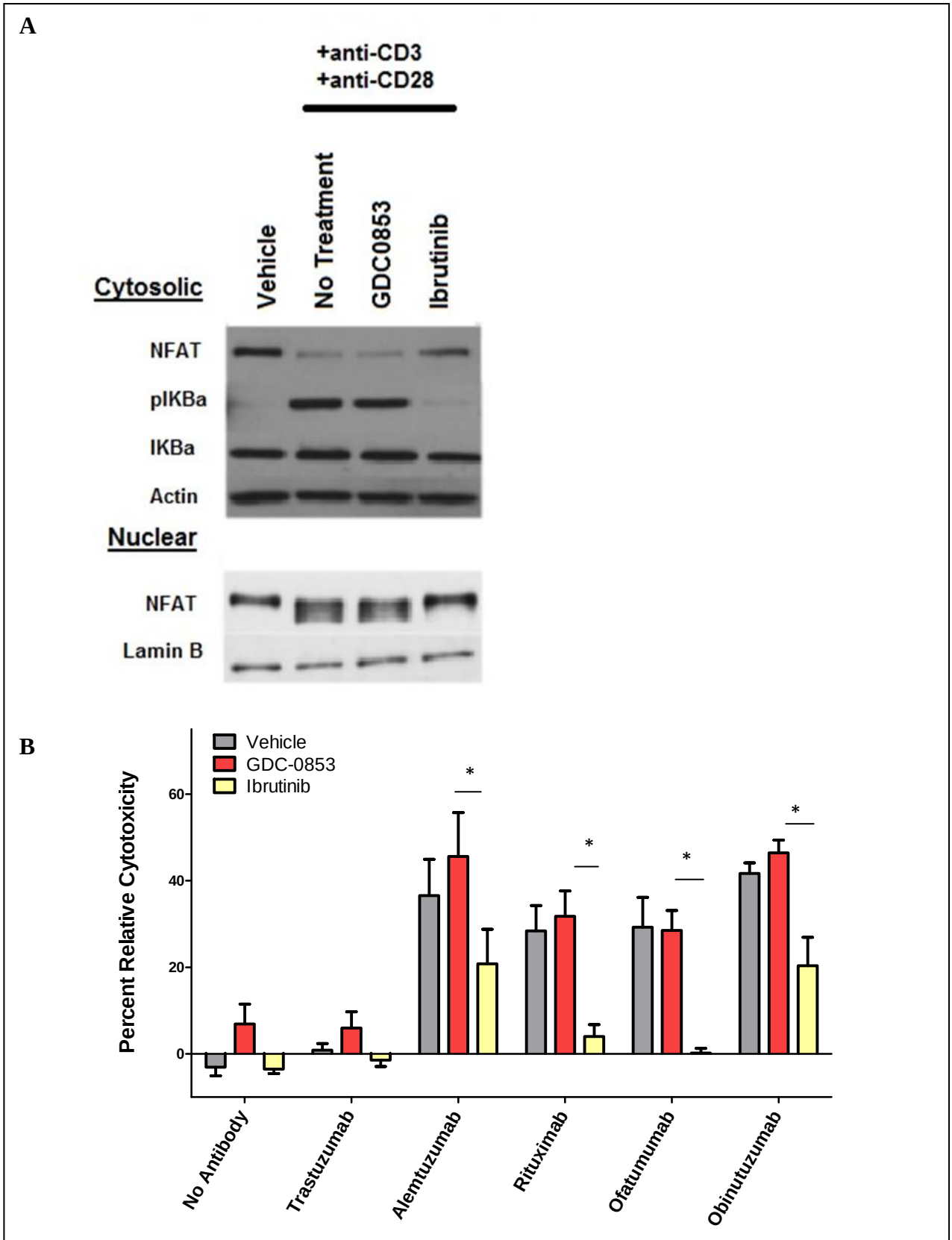

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